Apoptosis and Proliferation in Hepatocarcinogenesis Related to Cirrhosis

Young Nyun Park, M.D.[1]
Kwang Jo Chae, B.S.[1]
Young Bae Kim, M.D.[2]
Chanil Park, M.D.[1]
Neil Theise, M.D.[3]

[1] Department of Pathology and Brain, Korea 21 Project for Medical Science, Yonsei University College of Medicine, Seoul, Korea.

[2] Department of Pathology, Ajou University School of Medicine, Suwon, Korea.

[3] Department of Pathology, Tisch Hospital, New York University Medical Center, New York, New York.

Presented in part at the XXIII International Congress of the International Academy of Pathology, Nagoya, Japan, October 16, 2000.

Supported by Korean Research Foundation Grant (KRF-2000- 015-FP0028).

Address for reprints: Neil Theise, M.D., Room 461, Department of Pathology, Tisch Hospital, New York University Medical Center, 560 First Avenue, New York NY 10016; Fax: (212) 263-7916; E-mail: neil.theise@med.nyu.edu

Received August 13, 2001; revision received August 13, 2001; accepted August 20, 2001.

DOI 10.1002/cncr.10126

BACKGROUND. Dysplastic nodules (DNs) recently have been identified as preneoplastic lesions of hepatocellular carcinoma (HCC). To test an alternative hypothesis regarding DN development, in which we have suggested that DNs develop as an infiltrating clonal expansion in advance of, or parallel to cirrhosis, the authors investigated the rates of apoptosis and proliferation in human hepatocarcinogenesis.

METHODS. The authors performed terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay and proliferation cell nuclear antigen (PCNA) staining in 11 low-grade DNs, 8 high-grade DNs including 3 cases with HCC subnodules, 10 small HCCs, and 29 cases of surrounding cirrhotic nodules. Hepatocellular carcinoma subnodules were present in three cases of high DNs. They determined TUNEL-labeling indices (LIs) and PCNA-LIs as the percentage of positive hepatocyte nuclei per 500 randomly counted cells.

RESULTS. TUNEL-LIs (mean ± standard deviation) were 0.8 ± 0.82 in cirrhotic nodules, 1.0 ± 0.98 in low-grade DNs, 3.0 ± 4.33 in high-grade DNs, 8.7 ± 7.71 in HCC subnodules of high-grade DNs, and 3.2 ± 3.58 in small HCCs. The peak values of apoptotic activity were higher in high-grade DNs and HCCs than in low-grade DNs and cirrhotic nodules. Each case of low-grade DN showed a low to medium level of apoptotic activity when compared with those of the four surrounding cirrhotic nodules. The PCNA-LIs were 2.6 ± 1.35 in cirrhotic nodules, 4.5 ± 2.31 in low-grade DNs, 15.3 ± 10.50 in high-grade DNs, 25.4 ± 5.25 in HCC subnodules of high-grade DNs, and 34.9 ± 15.70 in small HCCs. The peak values gradually increased, although only HCC showed significantly elevated proliferation activity. The differences of PCNA-LIs and TUNEL-LIs, measured in each case, were 1.7 ± 1.89 in cirrhotic nodules, 3.6 ± 2.43 in low-grade DNs, 7.9 ± 5.69 in high-grade DNs, 16.2 ± 2.87 in HCC subnodules of high-grade DNs, 28.2 ± 13.97 in small HCCs. At all stages of hepatocarcinogenesis, the rates of cell proliferation were higher than apoptosis, allowing a preferential net gain of (pre)neoplastic cells, and it was significantly increased in small HCCs. In regenerative cirrhotic nodules, 14% (4 cases) showed higher rates of apoptosis than proliferation.

CONCLUSIONS. The regulation/dysregulation of apoptosis of (pre)neoplastic cells as well as of proliferation may play an important role in the process of hepatocarcinogenesis. ***Cancer* 2001;92:2733–8.** *© 2001 American Cancer Society.*

KEYWORDS: apoptosis, proliferation, dysplastic nodule, hepatocellular carcinoma.

Dysplastic nodules (DNs) have been identified as precancerous lesions of hepatocellular carcinomas (HCCs), arising in human chronic liver disease.[1,2] How DNs actually form has not yet been firmly established. One early view suggested that an ordinary regenerative nodule in cirrhosis becomes more rapidly proliferative, therefore becoming larger. In turn, the growing nodule is at greater risk for carcinogenic "hits," thereby giving rise to atypia and carcinoma.[3]

Although simple, this hypothesis does not take into consideration three known findings about DNs. First, they can be found in livers in advance of cirrhosis and therefore do not always arise from preexistent regenerative nodules.[4–6] Second, the presence of many intact portal tracts in most DNs, which have not yet been demonstrated to fully reconstitute after scarring and injury, suggests that they must be preexistent to the formation of the DN, because it is unlikely that a small cirrhotic nodule with few if any portal tracts could enlarge to a nodule with many portal tracts. Third, some DNs have been demonstrated to be clonal lesions, and therefore already neoplastic, not hyperplastic phenomena.[7–9]

To account for these features, we have suggested an alternative process of DN development,[5,10,11] in which DNs develop as an spreading clonal expansion in advance of or parallel to cirrhosis. We previously reported that this clonal expansion is resistant to the scarring affecting the adjacent liver tissue so that, as the rest of the liver around it becomes cirrhotic, the neoplastic expansion takes on the macroscopic appearance of a large cirrhotic nodule.[12]

On the basis of this alternate hypothesis, it was predicted that the spreading clonal expansion preceded the development of cirrhosis and therefore could perhaps take years to develop. Thus, it is possible that DNs could have relatively low proliferative rates, rather than the high proliferative rates necessitated by the earlier model. In fact, this prediction was confirmed[13]: low-grade DNs and the background parenchyma of high-grade DNs, outside of atypical subnodules, had proliferation rates that were similar or lower than that of surrounding cirrhotic nodules. Careful analysis of other studies of proliferation[14–17] demonstrates the same finding, although most investigators do not separate atypical foci from background normal hepatocytes in high-grade DNs in performing their analyses, obscuring this effect.

If these hepatocytes have a survival advantage compared with hepatocytes in the surrounding liver, they would expand in a clustered fashion, although they are not rapidly proliferative. Two possibilities are that they might be resistent to the disease damaging the surrounding parenchyma or they might have impaired mechanisms of apoptosis. We tested this latter possibility by performing terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay and proliferation cell nuclear antigen (PCNA) staining in low-grade DNs, high-grade DNs, small HCCs, and the surrounding cirrhotic nodules.

MATERIALS AND METHODS

We used routinely processed histologic sections from cirrhotic livers removed at the time of orthotopic liver transplantation or liver resection. We selected 11 representative low-grade DNs, 8 high-grade DNs including 3 cases with HCC subnodules, 10 small HCCs, and 29 cases of surrounding cirrhotic nodules from 12 patients (11 males and 1 female; 8 Americans, 1 Asian American, and 3 Koreans). The patients did not receive any chemotherapy before surgery. All cases showed cirrhosis, and the underlying causes of cirrhosis were hepatitis B virus in five patients, hepatitis C virus in five patients, autoimmune hepatitis in one patient, and alpha1-antitrypsin deficiency in one patient. The DNs were classified according the standard criteria of an International Working Party.[1] The size of the lesions ranged from 0.8 to 1.5 cm (1.11 $\pm$ 0.266 cm, mean $\pm$ standard deviation [SD]) in low-grade DNs, from 0.8 cm to 1.7cm (1.35 $\pm$ 0.366 cm) in high-grade DNs, and from 0.9 to 2.2 cm (1.37 $\pm$ 0.464 cm) in small HCCs (Table 1).

An additional section of each nodule was stained by means of a biotin-streptavidin amplified method using monoclonal antibodies to PCNA (Biogenex, San Ramon, CA) to evaluate the proliferation activity, and the immunohistochemical staining was performed as previously described.[13] We applied TUNEL assay for the detection of apoptosis using the apotag kit (Oncor, Gaithersburg, MD). Paraffin embedded sections of the liver were subjected to TUNEL assay according to the manufacturer's protocol.

In each specimen, the PCNA labeling indices (PCNA-LIs) and the TUNEL labeling indices (TUNEL-LIs) were determined in the DNs, HCCs, and the four surrounding cirrhotic nodules on the same slide. In each nodule and subnodule, 500 nuclei were randomly selected using a monocular eye-piece grid and a mechanical stage. The PCNA-LIs and TUNEL-LIs were expressed as the percentage of positive nuclei per total nuclei counted.

Statistical analyses were performed using Mann–Whitney *U* test.

RESULTS

We observed TUNEL and PCNA positive staining specifically only in the nuclei. In cirrhotic nodules and DNs, most of the TUNEL positive hepatocytes undergoing apoptosis were morphologically intact in the hematoxylin and eosin stain. In addition, some of the TUNEL positive cells in HCC showed the characteristic morphologic features of apoptosis. Three of the high-grade DNs had subnodules of HCC as a nodule in a nodule pattern (Fig. 1).

TABLE 1
Clinical and Pathologic Findings of the Patients

Patient no.	Age	Gender	Underlying cause of cirrhosis	Pathologic diagnosis (cm)
1	42	M	Hepatitis C virus	Low-grade DN (1.4)
2	54	M	Hepatitis C virus	HCC (0.9)
3	63	M	Hepatitis C virus	Low-grade DNs (0.9, 1.0, 1.2, 1.5)
4	40	M	Hepatitis C virus	HCC (1.5)
5	50	M	Hepatitis C virus	HCCs (1.0, 1.3, 1.5, 2.0)
6	47	M	Hepatitis B virus	HCC (2.2)
7	58	M	Hepatitis B virus	High-grade DNs (1.3, 1.8)
8	56	M	Hepatitis B virus	HCC (0.9)
9	53	M	Hepatitis B virus	High-grade DNs (0.8, 0.9), High-grade DNs with HCC subnodule (1.5, 1.6)
10	50	M	Hepatitis B virus	Low-grade DNs (0.8, 0.8, 0.9, 1.0)
11	43	F	Autoimmune hepatitis	High-grade DN (1.2)
12	56	M	α1-antitrypsin deficiency	Low-grade DNs (1.2, 1.5) High-grade DN with HCC subnodule (1.7) HCCs (0.9, 1.5)

M: male; DN: dysplastic nodule; HCC: hepatocellular carcinoma; F: female.

We found that TUNEL-LIs (mean ± standard deviation) were 0.8 ± 0.82 in cirrhotic nodules, 1.0 ± 0.98 in low-grade DNs, 3.0 ± 4.33 in high-grade DNs, 8.7 ± 7.71 in HCC subnodules of the high-grade DNs, and 3.2 ± 3.58 in small HCCs (Fig. 2). The peak values of apoptotic activity were higher in high-grade DNs, HCC subnodules, and small HCCs than in the low-grade DNs and cirrhotic nodules. The TUNEL-LIs of the low-grade DNs were of a low to medium level, compared with those of the four surrounding cirrhotic nodules in each case, although the rates of apoptosis in the low-grade DNs were not significantly lower than those of surrounding regenerative nodules.

We found that PCNA-LIs were 2.6 ± 1.35 in cirrhotic nodules, 4.5 ± 2.31 in low-grade DNs, 15.3 ± 10.50 in high-grade DNs, 25.4 ± 5.25 in HCC subnodules of high-grade DNs, and 34.9 ± 15.70 in small HCCs (Fig. 3). The peak values gradually increased according to the progression of multistep hepatocarcinogenesis, although only HCC showed significantly elevated proliferation activity ($P < 0.05$).

At all stages of hepatocarcinogenesis, with the exception of one case of low-grade DN, the rates of cell proliferation were higher than apoptosis allowing a preferential net gain of (pre)neoplastic cells. The differences of PCNA-LIs and TUNEL-LIs, measured in each case, were 1.7 ± 1.89 in cirrhotic nodules, 3.6 ± 2.43 in low-grade DNs, 7.9 ± 5.69 in high-grade DNs, 16.2 ± 2.87 in HCC subnodules of high-grade DNs, and 28.2 ± 13.97 in HCCs (mean ± SD; Fig. 4). These differences gradually increased and resulted in a significantly increased net gain in small HCCs. In regenerative cirrhotic nodules, 4 cases (14%) showed higher rates of apoptosis than proliferation.

DISCUSSION

Excessive cell accumulation during carcinogenesis can result not only from increased cellular proliferation, but also from diminished cell death. In experimental hepatocarcinogenesis in a rodent model, initiation is negatively regulated by the rate of hepatocyte apoptosis. Clonal expansion of the initiated hepatocytes occurs during the stage of promotion, and this expansion is caused by a selective increase in cell proliferation and by a selective decrease in apoptosis of preneoplastic hepatocytes. Several studies have now demonstrated that several promoting agents selectively inhibit apoptosis in preneoplastic hepatocytes and withdrawal of a promoting agent may cause a substantial increase in the apoptosis of hepatocytes and preneoplastic lesions.[18–21] During the stage of progression, the rate of apoptosis appears to increase with increasing cell replication.[22–24] The abrogation of apoptosis that results in survival advantage has been demonstrated in other tumors; for example, apoptosis is inhibited early in the dysplasia-carcinoma sequence of Barrett esophagus.[25]

Recently, DNs have been identified as premalignant lesions in the multistep process of hepatocarcinogenesis in humans. We have suggested an alternative hypothesis regarding this process of DN development.[5,10,11] The steps of this hypothetical process are as follows:

1. A clonal expansion of hepatocytes after the earliest carcinogenic events in response to any diffuse injury of the liver, which, in turn, leads to increased hepatocyte turnover;
2. These early hits lead to a clonal expansion of

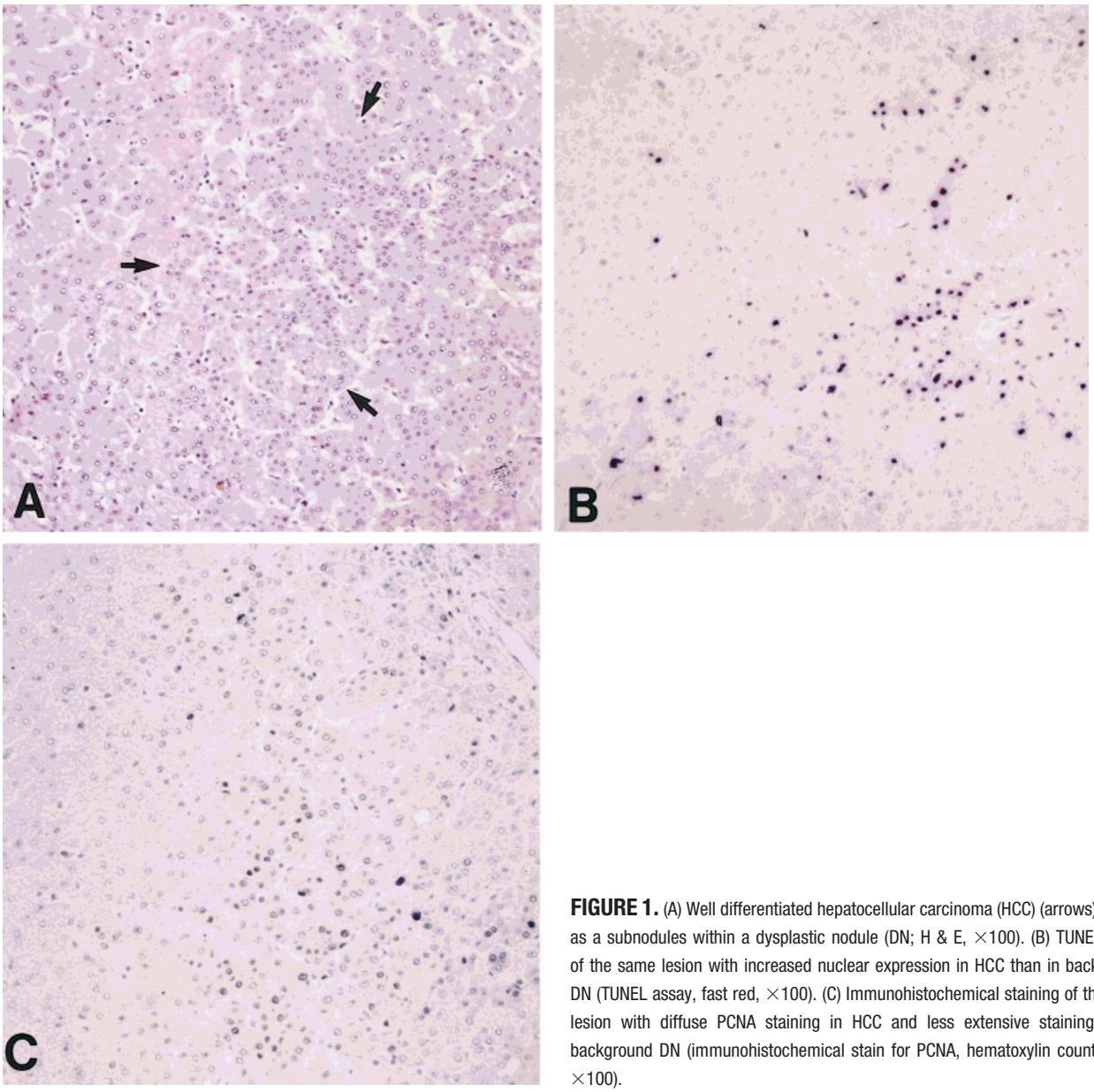

FIGURE 1. (A) Well differentiated hepatocellular carcinoma (HCC) (arrows) arising as a subnodules within a dysplastic nodule (DN; H & E, ×100). (B) TUNEL assay of the same lesion with increased nuclear expression in HCC than in background DN (TUNEL assay, fast red, ×100). (C) Immunohistochemical staining of the same lesion with diffuse PCNA staining in HCC and less extensive staining in the background DN (immunohistochemical stain for PCNA, hematoxylin counterstain, ×100).

hepatocytes that spreads around adjacent portal structures rather than displacing them;

3. As the rest of the liver becomes scarred, progressing to the later stages of disease and eventually cirrhosis, the island of clonal hepatocytes, if resistent to the scarring affecting the rest of the liver, would remain intact—an island of relatively preserved hepatic parenchyma made up of neoplastic, clonal hepatocytes;
4. With establishment of cirrhosis in the adjacent liver tissue, the clonal expansion takes on the appearance of a large cirrhotic nodule;
5. Having already undergone the earliest transforming events of hepatocarcinogenesis, the clonal, hepatocyte expansion remains at increased risk for later developments, and, thus, the lesion becomes the likeliest site of full malignant transformation.

On the basis of this alternate hypothesis, we tested the difference between cell proliferation and death by way of PCNA staining and TUNEL assay in low-grade DNs, high-grade DNs, small HCCs, and surrounding cirrhotic nodules. The PCNA data confirmed earlier studies that found that low-grade DNs did not have increased proliferative rates as compared with the surrounding regenerative nodules, and increases in proliferation only occurred with the development of atypia.[13,17] Apoptosis appeared diminished in low-

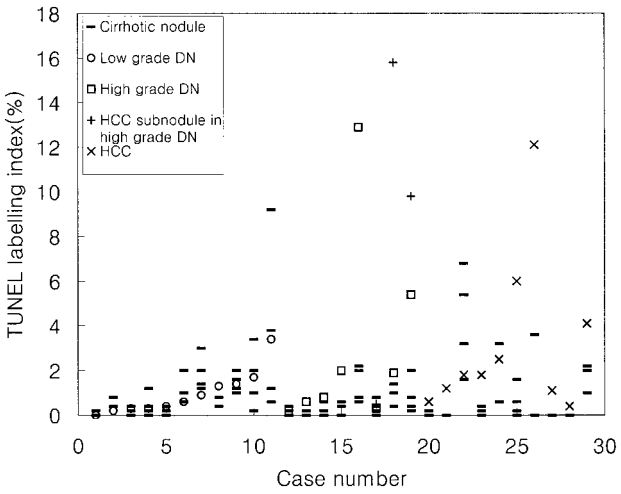

FIGURE 2. Results of TUNEL assay in human multistep hepatocarcinogenesis. TUNEL: terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling; DN: dysplastic nodule; HCC: hepatocellular carcinoma.

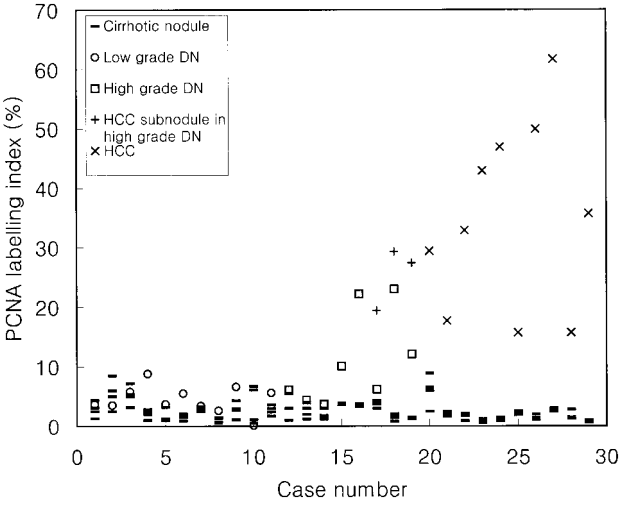

FIGURE 3. Results of immunohistochemical staining for PCNA in human multistep hepatocarcinogenesis. PCNA: cell proliferation nuclear antigen; DN: dysplastic nodule; HCC: hepatocellular carcinoma.

grade DNs, only increasing with the emergence of atypia. The rates of apoptosis in low-grade DNs were not significantly lower than those of the surrounding regenerative nodules; however, the ratio of apoptosis to proliferation was decreased in DNs, indicating that the hepatocyte populations, as expected, did have a survival advantage when compared with non-DN hepatocytes. Thus, the early stages of human hepatocarcinogenesis are marked by diminished apoptosis, compared with proliferation, resulting in an early spreading expansion of neoplastic hepatocytes.

In this study, the net gain of hepatocytes was gradually increased through the multiple stages of

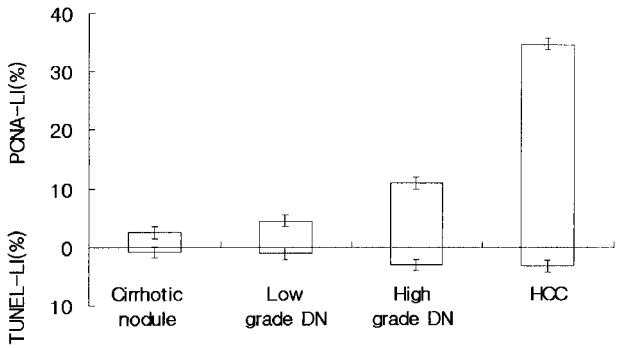

FIGURE 4. Difference of PCNA-labeling index and TUNEL-labeling index in human multistep hepatocarcinogenesis. TUNEL: terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling; PCNA: cell proliferation nuclear antigen; LI: labeling index; DN: dysplastic nodule; HCC: hepatocellular carcinoma. Means and standard deviation are given.

hepatocarcinogenesis. It significantly increased in small HCCs, despite the higher level of TUNEL-LI. These current findings showed a more rapid cell turnover of hepatocyte in HCC than DNs, and balance between proliferation and apoptosis is important in the progression of hepatocarcinogenesis. It also suggests that the homeostatic mechanisms of growth control are not totally destroyed in hepatocarcinogenesis or in HCCs.

REFERENCES

1. International Working Party. Terminology of nodular hepatocellular lesions. *Hepatology* 1995;22:983–93.
2. Hytiroglou P, Theise ND, Schwartz M, Mor E, Miller C, Thung SN. Macroregenerative nodules in a series of adult cirrhotic liver explants: issues of classification and nomenclature. *Hepatology* 1995;21:703–8.
3. Sakamoto M, Hirohashi S, Shimosato Y. Early stages of multistep hepatocarcinogenesis: adenomatous hyperplasia and early hepatocellular carcinoma. *Hum Pathol* 1991;22:172–8.
4. Furuya K, Nakamura M, Yamamoto Y, Togei K, Otsuka H. Macroregenerative nodule of the liver. A clinicopathologic study of 345 autopsy cases of chronic liver disease. *Cancer* 1988;61:99–105.
5. Theise ND, Lapook JD, Thung SN. A macroregenerative nodule containing multiple foci of hepatocellular carcinoma in a noncirrhotic liver. *Hepatology* 1993;17:993–6.
6. Matsuno Y, Hirohashi S, Furuya S, Sakamoto M, Mukai K, Shimosato Y. Heterogeneity of proliferative activity in nodule-in-nodule lesions of small hepatocellular carcinoma. *Jpn J Cancer Res* 1990;81:1137–40.
7. Tsuda H, Hirohashi S, Shimosato Y, Terada M, Hasegawa H. Clonal origin of atypical adenomatous hyperplasia of the liver and clonal identity with hepatocellular carcinoma. *Gastroenterology* 1988;95:1664–6.
8. Aihara T, Noguchi S, Sasaki Y, Nakano H, Monden M, Imaoka S. Clonal analysis of precancerous lesion of hepatocellular carcinoma. *Gastroenterology* 1996;111:455–61.

9. Ochiai T, Urata Y, Yamano T, Yamagishi H, Ashihara T. Clonal expansion in evolution of chronic hepatitis to hepatocellular carcinoma as seen at an X-chromosome locus. *Hepatology* 2000;31:615–21.
10. Theise N, Hytiroglou P, Thung S. Macroregenerative nodules and hepatocarcinogenesis. Il Friuli Medico. *Alpe Adria J Med* 1994;49:637–50.
11. Theise ND. Macroregenerative (dysplastic) nodules and hepatocarcinogenesis: theoretical and clinical considerations. *Semin Liver Dis* 1995;15:360–71.
12. Park YN, Yang CP, Cubukcu O, Thung SN, Theise ND. Hepatic stellate cell activation in dysplastic nodules: evidence for an alternate hypothesis concerning human hepatocarcinogenesis. *Liver* 1997;17:271–4.
13. Theise ND, Marcelin K, Goldfischer M, Hytiroglou P, Ferrell L, Thung SN. Low proliferative activity in macroregenerative nodules: evidence for an alternate hypothesis concerning human hepatocarcinogenesis. *Liver* 1996;16:134–9.
14. Le Bail B, Belleannee G, Bernard P-H, Saric J, Balabaud C, Bioulac-Sage P. Adenomatous hyperplasia in cirrhotic livers: histological evaluation, cellular density, and proliferative activity of 35 macronodular lesions in the cirrhotic explants of 10 adult French patients. *Hum Pathol* 1995;26:897–906.
15. Terasaki S, Terada T, Nakanuma Y, Nonomura A, Unoura M, Kobayashi K. Argyrophilic nucleolar organizer regions and alpha-fetoprotein in adenomatous hyperplasia in human cirrhotic livers. *Am J Clin Pathol* 1991;95:850–7.
16. Grigioni WF, D'Errico A, Bacci F, Gaudio M, Mazziotti A, Gozzetti G, et al. Primary liver neoplasms: evaluation of proliferative index using MoAb Ki67. *J Pathol* 1989;158:23–9.
17. Tiniakos DG, Brunt EM. Proliferating cell nuclear antigen and Ki-67 labeling in hepatocellular nodules: a comparative study. *Liver* 1999;19:58–68.
18. Schulte-Hermann R, Bursch W, Low-Baselli A, Wagner A, Grasl-Kraupp B. Apoptosis in the liver and its role in hepatocarcinogenesis. *Cell Biol Toxicol* 1997;13:339–48.
19. Hikita H, Vaughan J, Pitot HC. The effect of two periods of short-term fasting during the promotion stage of hepatocarcinogenesis in rats: the role of apoptosis and cell proliferation. *Carcinogenesis* 1997;18:159–66.
20. Bursch W, Lauer B, Timmermann-Trosiener I, Barthel G, Schuppler J, Schulte-Hermann R. Controlled death (apoptosis) of normal and putative preneoplastic cells in rat liver following withdrawal of tumor promoters. *Carcinogenesis* 1984;5:453–8.
21. Columbano A, Ledda-Columbano GM, Coni PP, Faa G, Liguori C, Santa Cruz G, et al. Occurrence of cell death (apoptosis) during the involution of liver hyperplasia. *Lab Invest* 1985;52:670–5.
22. Grasl-Kraupp B, Ruttkay-Nedecky B, Mullauer L, Taper H, Huber W, Bursch W, et al. Inherent increase of apoptosis in liver tumors: implications for carcinogenesis and tumor regression. *Hepatology* 1997;25:906–12.
23. Terada T, Nakanuma Y. Expression of apoptosis, proliferating cell nuclear antigen, and apoptosis-related antigens (bcl-2, c-myc, Fas, Lewis^y and p53) in human cholangiocarcinoma and hepatocellular carcinoma. *Pathol Int* 1996;46:764–70.
24. Hino N, Higashi T, Nouso K, Nakatsukasa H, Tsuji T. Apoptosis and proliferation of human hepatocellular carcinoma. *Liver* 1996;16:123–9.
25. Katada N, Hinder RA, Smyrk TC, Hirabayashi N, Perdikis G, Lun RJ, et al. Apoptosis is inhibited early in the dysplasia-carcinoma sequence of Barrett esophagus. *Arch Surg* 1997;132:728–33.